

# Signal transducer and activator of transcription 3-regulated sarcoendoplasmic reticulum $\text{Ca}^{2+}$ -ATPase 2 expression by prolactin and glucocorticoids is involved in the adaptation of insulin secretory response during the peripartum period

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## Abstract

During pregnancy, the maternal endocrine pancreas undergoes, as a consequence of placental lactogens and prolactin (PRL) action, functional changes that are characterized by increased glucose-induced insulin secretion. After delivery, the maternal endocrine pancreas rapidly returns to non-pregnant state, which is mainly attributed to the increased serum levels of glucocorticoids (GCs). Although GCs are known to decrease insulin secretion and counteract PRL action, the mechanisms for these effects are poorly understood. We have previously demonstrated that signal transducer and activator of transcription 3 (STAT3) is increased in islets treated with PRL. In the present study, we show that STAT3 expression and serine phosphorylation are increased in pancreatic islets at the end of pregnancy (P19). STAT3 serine phosphorylation rapidly returned to basal levels 3 days after delivery (L3). The expression of the sarcoendoplasmic

reticulum  $\text{Ca}^{2+}$ -ATPase 2 (SERCA2), a crucial protein involved in the regulation of calcium handling in  $\beta$ -cells, was also increased in P19, returning to basal levels at L3. PRL increased SERCA2 and STAT3 expressions and STAT3 serine phosphorylation in RINm5F cells. The upregulation of SERCA2 by PRL was abolished after STAT3 knockdown. Moreover, PRL-induced STAT3 serine phosphorylation and SERCA2 expression were inhibited by dexamethasone (DEX). Insulin secretion from islets of P19 rats pre-incubated with thapsigargin and L3 rats showed a dramatic suppression of first phase of insulin release. The present results indicate that PRL regulates SERCA2 expression by a STAT3-dependent mechanism. PRL effect is counteracted by DEX and might contribute to the adaptation of maternal endocrine pancreas during the peripartum period.

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## Introduction

In pancreatic  $\beta$ -cells, the most prominent effect attributed to Janus kinase/signal transducer and activator of transcription 5 (JAK/STAT5) signaling pathway is the functional adaptation induced by placental lactogens (PL) and prolactin (PRL) during pregnancy (Brelje *et al.* 1993, Nielsen *et al.* 1999). The biological effects exerted by PRL in the endocrine pancreas are characterized by a marked increase in pancreatic  $\beta$ -cell proliferation and sensitivity to glucose, together with a decrease in apoptosis (Parsons *et al.* 1992, Scaglia *et al.* 1997, Bonner-Weir 2001).

The elevated concentration of glucocorticoids (GCs) found during the late stage of pregnancy is believed to counteract the PRL-induced adaptation of endocrine pancreas, reestablishing the non-pregnant phenotype in pancreatic  $\beta$ -cells. It has been

demonstrated that dexamethasone (DEX), a synthetic glucocorticoid, reverses PRL-induced upregulation of islet function by inhibiting glucose metabolism (Shao *et al.* 2004), PDX-1 expression (Nasir *et al.* 2005), insulin secretion, and cell proliferation, and by increasing apoptosis (Weinhaus *et al.* 2000). Curiously, GCs have been reported to strongly synergize with PRL-induced STAT5 activation through distinct mechanisms involving the glucocorticoid receptor (Stocklin *et al.* 1996, Lechner *et al.* 1997, Wyzomierski *et al.* 1999). Therefore, a distinct mechanism activated by PRL, other than the activation of the JAK2/STAT5 pathway, is likely to be negatively modulated by GCs in pancreatic  $\beta$ -cells.

It is well known that the exocytosis of insulin-containing granules is tightly regulated by changes in cytosolic  $\text{Ca}^{2+}$  concentration. A disturbance in the  $\text{Ca}^{2+}$  sequestration by

the endoplasmic reticulum (ER), as a result of a defect in sarcoER  $\text{Ca}^{2+}$ -ATPase (SERCA) activity and expression, has been suggested as the cause of impaired insulin secretion in several animal models of glucose intolerance (Marie *et al.* 2001, Kulkarni *et al.* 2004), including the *db/db* mice (Roe *et al.* 1994). *db/db* Mice display obesity and type 2 diabetes, due to the absence of functional leptin receptors, the main activator of STAT3 in pancreatic  $\beta$ -cells (Shafir 1992, Morton *et al.* 1999). We have demonstrated that adult pancreatic islets, cultured with PRL, show an upregulation in STAT3 gene expression (Bordin *et al.* 2004). It is noteworthy that STAT3 knockout mice develop abnormal islet architecture and an impairment in the early phase of glucose-induced insulin secretion (Gorogawa *et al.* 2004) and glucose intolerance (Cui *et al.* 2004).

In the present study, we aimed to investigate whether the expression and phosphorylation status of STAT3 in pancreatic islets of pregnant and lactating rats correlates with the SERCA isoforms expression and, thus, with the insulin secretion pattern. We found that PRL induces SERCA2 expression in pancreatic  $\beta$ -cells through a STAT3-mediated mechanism. Additionally, we show that this phenomenon is inhibited by DEX.

## Materials and Methods

### Materials

General chemical reagents were obtained from Synth (Diadema, SP, Brazil). RPMI-1640 medium, trypsin, and fetal bovine serum (FBS) were from Cultilab (Campinas, SP, Brazil). BSA (fraction V), collagenase type V, Tris, dithiothreitol, luminol, p-coumaric acid, and thapsigargin were from Sigma. Rat PRL was purchased from Dr A F Parlow (Harbor University of California Los Angeles Medical Center, USA). DEX disodium phosphate was from Laboratórios Aché (São Paulo, SP, Brazil). The apparatus for SDS-PAGE was from Bio-Rad. The nitrocellulose membranes were purchased from Bioagency (São Paulo, SP, Brazil). Antibodies against STAT3 (sc483), STAT5 (sc835), pSTAT3-Tyr (sc8059), SERCA2 (sc8095), SERCA3 (sc8097),  $\alpha$ -tubulin (sc-8035), and HRP-conjugated and anti-goat were from Santa Cruz Technology (Santa Cruz, CA, USA). The antibody against pSTAT3-Ser (9134) was from Cell Signaling (Danvers, MA, USA). [ $^{125}\text{I}$ ]Insulin, HRP-conjugated anti-rabbit and anti-mouse antibodies were from Amersham-Pharmacia Biotech. X-ray sensitive films and chemicals were from IBF (Rio de Janeiro, RJ, Brazil). GoTaq DNA polymerase and ImProm-II reverse transcriptase were from Promega. PCR primers and phosphorothioate scrambled and antisense oligonucleotides were from IDT (Integrated DNA Technologies, Coralville, IA, USA). Opti-MEM, random primers, dNTP set, agarose, Trizol reagent, antibiotics, antimycotic, and Lipofectamine 2000 were from

Invitrogen. All plastics for cell culture were from TPP (Trasadingen, Switzerland).

### Animals and islet isolation

Two female rats and one male rat were housed together for 5 days. The presence of spermatozoa in the vaginal wash indicated day 0 of gestation. Immediately after delivery, the number of pups was adjusted to eight for each lactating mother. Pregnant and lactating rats were killed at 19 days post-coitus (P19) and 3 and 8 days *post partum* (L3 and L8 respectively). Virgin age-matched rats were used as the control group (CTL). Islets were isolated by collagenase digestion, as previously described (Bordin *et al.* 1995). All of the experiments involving animals were in accordance with the guidelines of the Brazilian College for Animal Experimentation.

### Insulin secretion

Cumulative insulin secretion was performed in pancreatic islets from female virgin rats after a 36-h culture. Previously to the culture, groups of 300 islets were transfected with antisense oligonucleotide (ASO) against STAT3 or treated only with Lipofectamine 2000. After culture, groups of five islets were first incubated for 30 min at 37 °C in Krebs-bicarbonate buffer containing 5.6 mM glucose and equilibrated with 95%  $\text{O}_2$ /5%  $\text{CO}_2$  (pH 7.4). The solution was then replaced by fresh Krebs-bicarbonate buffer and the islets were incubated for a further 60-min period with medium containing 2.8 or 16.7 mM glucose. After the incubation period, the supernatant was kept for insulin determination.

Insulin secretion in dynamic conditions was performed, as previously described (Bosqueiro *et al.* 2000). Briefly, groups of 20 islets were isolated from CTL, P19, and L3 rats, transferred to chambers, and perfused for 30 min with Krebs-bicarbonate containing 2.8 mM glucose. A group of islets isolated from P19 rats was also pre-incubated with 5  $\mu\text{M}$  thapsigargin for 20 min before the beginning of perfusion. The medium was then changed to Krebs-bicarbonate containing 16.7 mM glucose and the islets were perfused for an additional 30 min. The effluent was collected every 1 min from the min 20 until the end of the perfusion period. Since islets from P19 rats are usually larger than CTL and L3 islets, dynamic insulin secretion are expressed relative to insulin content. For this, the islets were sonicated in lysis buffer (70% ethanol and 0.2 M HCl) and incubated overnight at 4 °C. The insulin content of each sample was measured by RIA using rat insulin as a standard.

### RINm5F cells and isolated islets culture, treatment, and transfection

Islets and RINm5F cells were cultured in RPMI-1640 medium containing 11 mM glucose, 10% FBS, and penicillin and streptomycin in a humidified atmosphere with 5%  $\text{CO}_2$  at

37 °C. PRL and DEX were added to final concentrations of 500 ng/ml and 100 nM respectively. PRL was added to the medium 6 h prior to DEX. RINm5F cell treatment was carried out in serum-free medium containing 1% BSA, and islets in medium containing 10% FBS; 24 h after treatment, cells were used to western blot and islets for RNA extraction. For transfection, Opti-MEM containing 10 mM glucose plus a chimeric DNA–RNA 2′-O-methyl phosphorothioate oligonucleotide, previously mixed with Lipofectamine 2000, was added to RINm5F cells and isolated islets. CTL cells and islets were treated only with Lipofectamine. The oligonucleotides used for transfection were a scrambled oligonucleotide (S) with no effect on STAT3 expression, and an antisense oligonucleotide targeted to STAT3 (ASO). After 4 h, the medium was supplemented with RPMI containing 1% BSA with or without PRL. After 24 h, total protein was extracted and processed for western blotting. The sequences of the S and ASO oligonucleotides were 5′-GmCmUmGCTG-GAGCTGGmUmUmCmC-3′, and 5′-mGmCmAmC-GATCGATCCmCmAmUmG-3′ respectively where ‘m’ denotes RNA 2′-O-methyl nucleotides.

#### RT-PCR analysis

Total RNA was extracted from 24-h cultured islets using Trizol reagent. RT-PCR was performed as previously described (Bordin *et al.* 2004). The primer sets used in RT-PCR analysis, with their respective melting temperatures and product lengths, were: GLUT2: 5′-CATTGCTGGAAGAAGCGTATCAG-3′ and 5′-GAGACCTTCTGCTCAGTCGACG-3′, 55 °C, 408 bp; SUR1: 5′-TTCCACATCCTGGTCACACCG-3′ and 5′-AGAAGGAGCGAGGACTTGCCAC-3′, 60 °C, 425 bp; SERCA2: 5′-CTCTGGGTCAATCTGGTGACGG-3′ and 5′-TGCCATTGTTCATCGGATACGG-3′, 58 °C, 324 bp; and RPL37a: 5′-CAAGAAGGTCGGGATCGTCG-3′ and 5′-ACCAGGCAAGTCTCAGGAGGTG-3′, 57 °C, 290 bp. The RNAs used for RT-PCR analysis were obtained from three or four independent experiments.

#### Western blot analysis

Freshly isolated pancreatic islets and RINm5F cells were homogenized in 100 µl of solubilization buffer and processed for protein extraction and western blot, as previously described (Anhê *et al.* 2006). Before incubation with the primary antibody, the membranes were blocked with blocking buffer (5% nonfat dried milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20) for 2 h at 22 °C. The membranes were incubated for 4 h at 22 °C with the primary antibody diluted in blocking buffer with 3% nonfat dried milk. After being washed for 30 min in blocking buffer without milk, the membranes were incubated with peroxidase-conjugated secondary antibody for 1 h, and processed for chemiluminescence to visualize the immunoreactive bands. Quantitative analysis of the blots was performed by Scion Image software. When the primary antibody was a goat

polyclonal, nonfat dried milk was replaced by 1% gelatin in all incubations.

#### Statistical analysis

The results were expressed as the mean  $\pm$  S.E.M. The results were compared using ANOVA followed by the Tukey–Kramer test or Student’s unpaired *t*-test. *P* values <0.05 indicated a significant difference.

## Results

#### STAT3 expression and phosphorylation in pancreatic islets during pregnancy and lactation

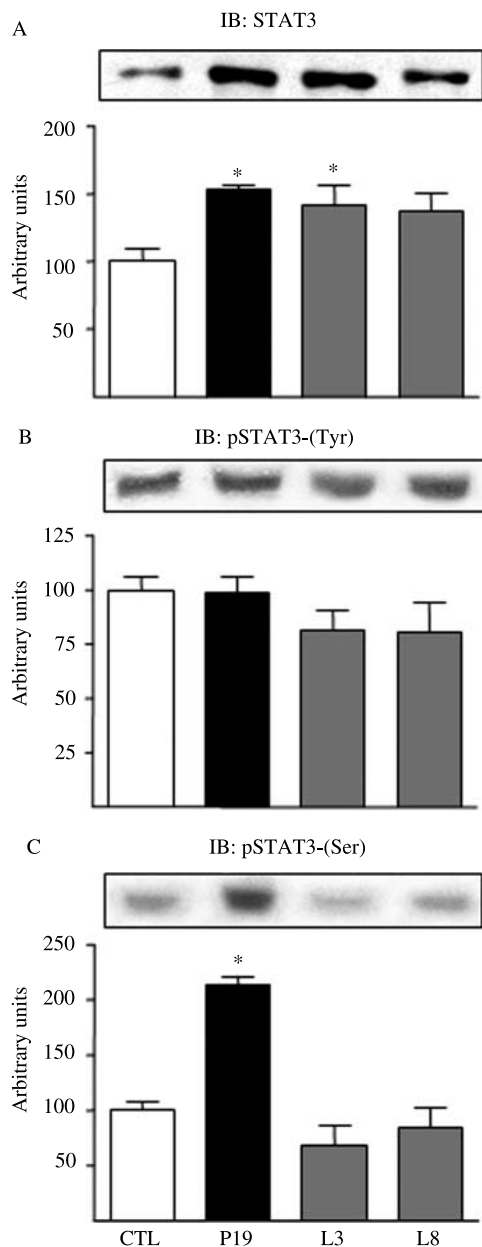
Our results show that the protein content of STAT3 is increased in pancreatic islets at the end of the gestational period (1.5-fold for P19 compared with CTL; *P*<0.05). This increase persisted until day 3 of lactation (1.4-fold for L3 compared with CTL; *P*<0.05) returning to control values on day 8 (L8; Fig. 1A). Tyrosine phosphorylation of STAT3 was not altered throughout the referred periods (Fig. 1B). STAT3 serine phosphorylation was increased in pancreatic islets from late pregnant rats (2.1-fold for P19 compared with CTL; *P*<0.05). The higher levels of STAT3 serine phosphorylation were temporally limited to pregnancy and returned to CTL values in L3 rats (Fig. 1C).

#### Dynamic insulin secretion by pancreatic islets from pregnant and lactating rats

In islets from pregnant and virgin rats, the introduction of 16.7 mM glucose in the perfusion medium induced a biphasic insulin secretory response characterized by a prominent and transient release (first phase) followed by a secondary rise (Fig. 2). As expected, overall secretion from pregnant rat islets during the stimulatory period was higher than that of the control. After parturition, total insulin secretion, measured over the stimulatory period (31–60 min), returned to values found in non-pregnant rat islets. However, the first-phase release from islets from lactating rats at L3 was abolished (*P*<0.05 compared with CTL and P19).

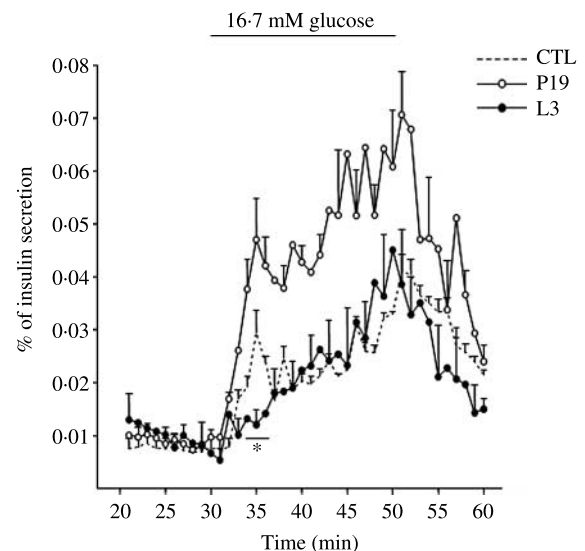
#### SERCA2 and SERCA3 expressions in pancreatic islets during pregnancy and lactation and participation of SERCA in the insulin secretion during pregnancy

The protein content of SERCA3 was similar in pancreatic islets from P19, L3, and CTL (Fig. 3A). However, SERCA2 was significantly increased in pancreatic islets from P19 rats, returning to CTL values in L3 rats (2.7-fold for P19 compared with CTL; *P*<0.05; Fig. 3B). In order to clarify the role of this SERCA2 in the dynamic feature of insulin secretion during pregnancy, we treated pancreatic islets from P19 rats with thapsigargin, an irreversible SERCA inhibitor.



**Figure 1** STAT3 expression, serine, and tyrosine phosphorylation in pancreatic islets from pregnant and lactating rats. Pancreatic islets were isolated from pregnant (P19) and lactating (L3 and L8) rats. Protein extracts were processed for western blotting for STAT3 (A;  $n=5$ ), pSTAT3-Tyr (B;  $n=5$ ) and pSTAT3-Ser (C;  $n=8$ ). The bars represent the means  $\pm$  S.E.M. of the values determined by optical densitometry. \* $P<0.05$  versus CTL.

Figure 3C shows that pharmacological blockade of SERCA in pancreatic islets from P19 rats resulted in a significant reduction of the first phase of insulin secretion ( $P<0.05$ ). In contrast, the secondary rise in insulin secretion from P19 islets treated with thapsigargin showed a tendency to be higher than P19 islets that were pre-incubated only with glucose.



**Figure 2** Dynamic insulin secretion by pancreatic islets from pregnant and lactating rats. Groups of 20 islets were isolated from CTL, P19, and L3 rats, transferred to chambers, and perfused with Krebs-bicarbonate containing 2.8 mM glucose for the initial 30 min. The medium was replaced by a solution containing 16.7 mM glucose for an additional 30 min of perfusion. The dotted line represents insulin secretion from CTL rat islets. Continuous lines with open and black circles represent insulin secretion from P19 and L3 rat islets respectively. Mean values ( $\pm$  S.E.M.) refer to four individual experiments. \* $P<0.05$  versus CTL.

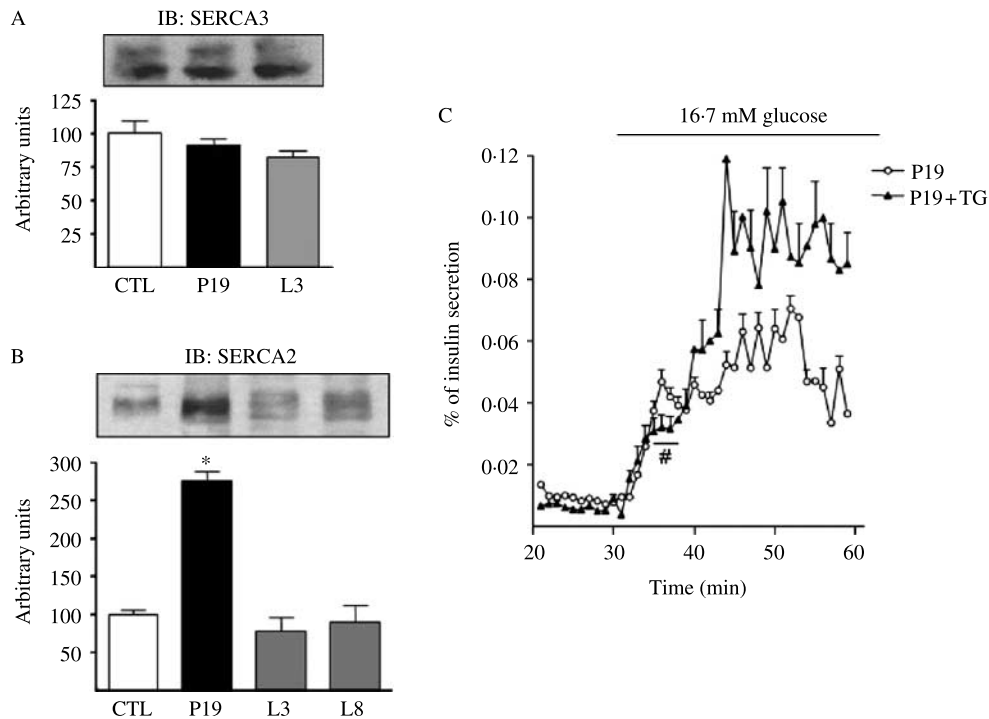
#### *Specificity and efficiency of the phosphorothioate antisense oligonucleotide targeted to STAT3*

Western blot analysis in RINm5F cells transfected with antisense phosphorothioate oligonucleotide targeted to STAT3 (ASO) revealed that this oligo efficiently reduces STAT3 protein content without any interference in STAT5 and  $\alpha$ -tubulin expressions. Additionally, the effect of ASO is not due to a non-specific effect, since the transfection with the scrambled control oligo (S) did not cause any disruption in STAT3 expression (Fig. 4A). As observed in pancreatic islets from STAT3-knockout mice (Gorogawa *et al.* 2004), GLUT2 (0.63-fold for ASO compared with CTL;  $P<0.05$ ) and SUR1 (0.60-fold for ASO compared with CTL;  $P<0.05$ ) mRNA expressions were decreased in RINm5F cells transfected with ASO (Fig. 4B and C respectively).

RINm5F cells treated with PRL for 24 h displayed an increase in STAT3 protein content (1.9-fold compared with CTL;  $P<0.05$ ). Transfection with ASO reduced STAT3 content (0.38-fold compared with CTL;  $P<0.05$ ) and abolished the PRL-induced increase in STAT3 (Fig. 4D).

#### *Modulation of SERCA2, but not SERCA3, by PRL in RINm5F cells and the participation of STAT3*

Figure 5A shows that PRL induced an increase in the SERCA2 content of RINm5F cells (1.59-fold compared with CTL;  $P<0.05$ ), while transfection with ASO reduced the SERCA2



**Figure 3** Participation of SERCA in insulin secretion during pregnancy and its expression in pancreatic islets from pregnant and lactating rats. Pancreatic islets were isolated from pregnant (P19) and lactating (L3 and L8) rats. Protein extracts were processed for western blot detection of SERCA3 (A;  $n=3$ ) and SERCA2 (B;  $n=4$ ). Groups of 20 islets were isolated from P19 rats and pre-incubated with (P19+TG) or without (P19) 5  $\mu$ M thapsigargin for 20 min before the beginning of perfusion. After this period, the islets were transferred to chambers and perfused with Krebs-bicarbonate containing 2.8 mM glucose for the initial 30 min. The medium was replaced by a solution containing 16.7 mM glucose for an additional 30 min of perfusion. Open circles represent P19 islets and black triangles represent P19 islets pre-incubated with TG (C;  $n=4$ ). The bars and the lines represent the means  $\pm$  S.E.M. of the values. \* $P<0.05$  versus CTL; # $P<0.05$  versus P19 in the absence of TG.

content (0.46-fold compared with CTL;  $P<0.05$ ). The upregulation of SERCA2 was not observed in RINm5F cells treated with PRL and previously transfected with ASO targeted to STAT3. The effect of PRL on RINm5F cells was specific for SERCA2 since no differences were found in SERCA3 expression (Fig. 5B). Figure 5C shows that the knockdown of STAT3 expression prompts an increase in the 1-h cumulative insulin secretion induced by glucose ( $P<0.05$  versus CTL islets with 16.7 mM glucose), without any effect on the basal (2.8 mM glucose) insulin secretion.

#### Combined effect of DEX and PRL on STAT3 expression and phosphorylation in RINm5F cells

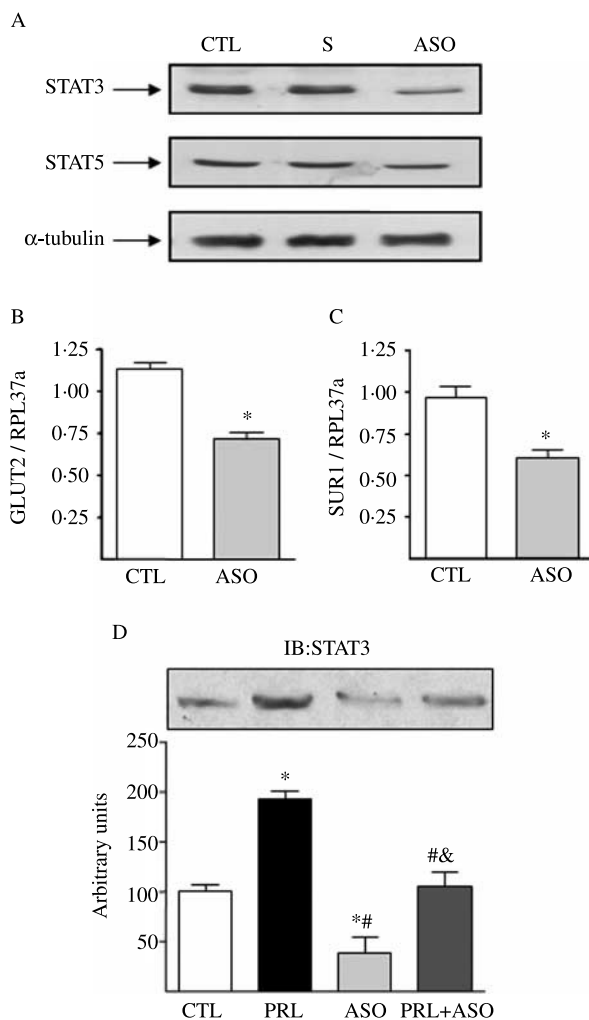
Figure 6A shows that PRL alone or in combination with DEX promoted a similar increase in the STAT3 content in RINm5F cells (1.95-fold for PRL and 2.00-fold for DEX + PRL when compared with CTL;  $P<0.05$ ). DEX alone did not alter STAT3 expression in RINm5F cells. Tyrosine phosphorylation of STAT3 was not altered by PRL, DEX, or the combination of both agents (Fig. 6B).

STAT3 serine phosphorylation was increased in RINm5F cells treated with PRL (1.30-fold compared with CTL;  $P<0.05$ ). DEX alone, or in combination with PRL, reduced the levels of STAT3 serine phosphorylation (0.60-fold for DEX and DEX+PRL compared with CTL;  $P<0.05$ ; Fig. 6C).

#### Combined effect of DEX and PRL on SERCA2 protein and mRNA expression in RINm5F cells and cultured rat pancreatic islets

Figure 7A shows that PRL-induced SERCA2 expression in RINm5F cells was abolished by the presence of DEX. Additionally, DEX alone promoted a reduction in SERCA2 content in RINm5F cells (0.75-fold compared with CTL;  $P<0.05$ ). Figure 7B shows that PRL also induced an increase in SERCA2 mRNA in cultured rat pancreatic islets (1.20-fold compared with CTL;  $P<0.05$ ). This effect was also counteracted by the addition of DEX to the culture medium and, in this case, DEX alone did not reduce SERCA2 expression (Fig. 7B).





**Figure 4** STAT3 knockdown in RINm5F cells treated with prolactin. RINm5F cells were transfected with a phosphorothioate oligonucleotide targeted to STAT3 (ASO) and with a scrambled oligonucleotide with no effect on STAT3 expression (S). CTL cells were treated only with the Lipofectamine. After 24 h from the transfection, the cells were processed for western blot detection of STAT3, STAT5, and  $\alpha$ -tubulin (A) and for RT-PCR of GLUT2 (B;  $n=7$ ) and SUR1 (C;  $n=5$ ) genes. After the transfection, a distinct group of cells (ASO and CTL), were transferred to a 1% BSA medium in the presence or in the absence of PRL. After 24 h, the cells were processed for western blot detection of STAT3 (D;  $n=7$ ). The bars represent the means  $\pm$  S.E.M. of the values determined by optical densitometry. \* $P<0.05$  versus CTL; # $P<0.05$  versus PRL and & $P<0.05$  versus ASO.

## Discussion

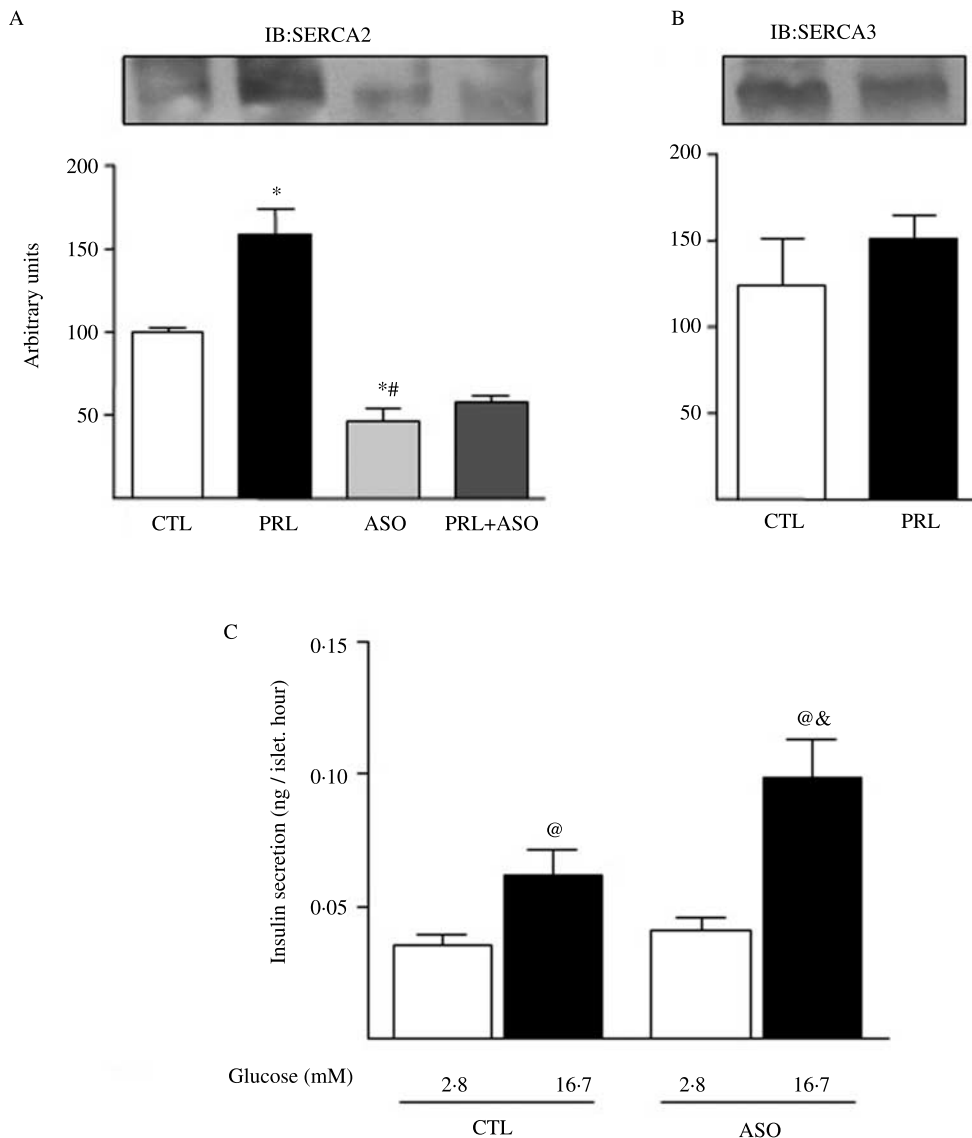
We have previously demonstrated that *in vitro* treatment of pancreatic islets with PRL increases STAT3 expression (Bordin *et al.* 2004). Attempting to investigate the participation of STAT3 in a physiological situation in which the high levels of PRL are determinant for the adaptation of the endocrine pancreas, we first examined STAT3 expression and

phosphorylation status in islets from pregnant and lactating rats. STAT3 expression was increased in pancreatic islets from P19 and L3 rats. Since PRL serum levels remain high during the transition from late pregnancy to early lactation (Kawai & Kishi 1999), it is reasonable to assume that modulation of STAT3 expression during the peripartum period may be due to PRL action. We also found that serine, but not tyrosine basal phosphorylation of STAT3, is increased in the pancreatic islets of late pregnant rats. Thus, the current study aimed to clarify the putative participation of STAT3 and its serine phosphorylation in the physiology of pancreatic islets during pregnancy.

The increase in STAT3 serine phosphorylation was also detected in RINm5F cells treated with PRL. Taken together, the *in vivo* and *in vitro* data of the present study strongly indicate that PRL may modulate not only STAT3 expression, but also its serine phosphorylation. In accordance to our results, Neilson and collaborators have recently shown that PRL induces direct activation of STAT3 after a 24-h treatment (Neilson *et al.* 2007). Interestingly, insulin has also been reported to stimulate STAT3 serine phosphorylation (Ceresa & Pessin 1996). PRL increases insulin secretion from isolated pancreatic islets (Sorenson *et al.* 1993, Crepaldi *et al.* 1997), and high serum insulin levels present in late pregnancy return to normal levels on day 1 after delivery (Kawai & Kishi 1999). Thus, the hypothesis that PRL increases STAT3 serine phosphorylation indirectly through the modulation of insulin secretion must also be considered to explain the results showed herein.

Although it is classically recognized that tyrosine phosphorylation of STAT3 is a prerequisite for the activation of gene transcription (Aaronson & Horvath 2002), the role of serine phosphorylation in the regulation of STAT3 activity remains controversial. Recent evidence suggests that STAT3 serine phosphorylation, which occurs within a mitogen activated protein kinase (MAPK) consensus site (Ser727), has a positive role in STAT3 transcriptional activation (Shen *et al.* 2004, Kojima *et al.* 2005, Sun *et al.* 2006). Indeed, extracellular signal-regulated protein kinases (ERKs) activation targets STAT3 serine phosphorylation (Kuroki & O'Flaherty 1999, O'Rourke & Shepherd 2002). We have previously demonstrated that PRL activates ERK1/2 *in vitro* in isolated islets (Amaral *et al.* 2003) and *in vivo* in islets from pregnant rats (Amaral *et al.* 2004). Therefore, we may speculate that PRL may target STAT3 serine phosphorylation in pancreatic islets by activation of ERK1/2. This can occur by a direct action of PRL or as the consequence of the increased insulin circulating levels, since insulin-induced STAT3 serine phosphorylation is ERK1/2 dependent (Ceresa *et al.* 1997).

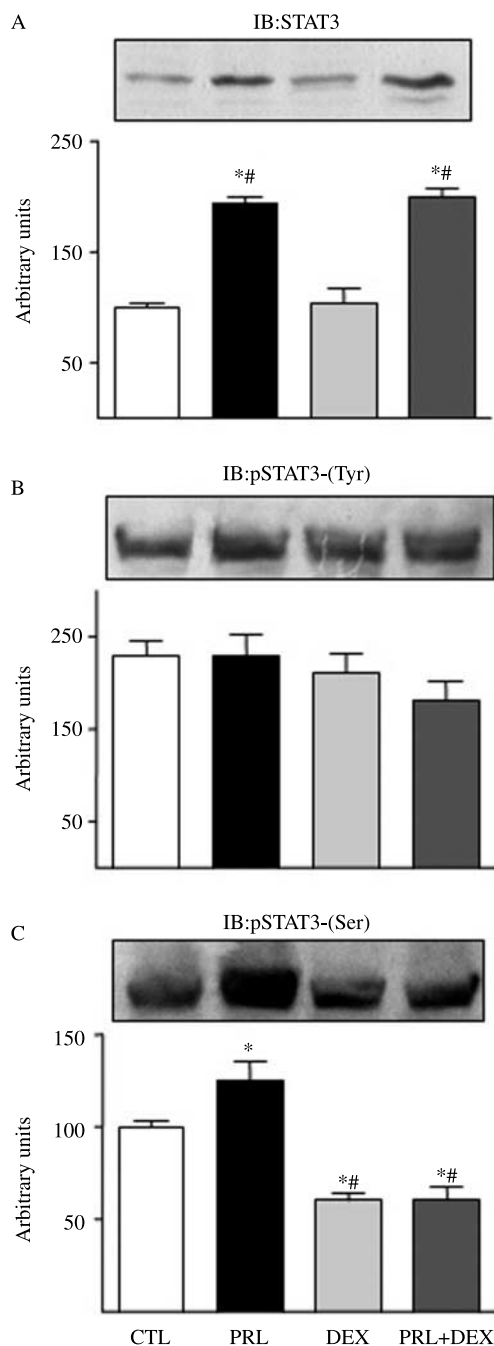
The dynamics of insulin secretion by pancreatic islets from pregnant rats showed that, during pregnancy, there is a marked increase in glucose response. This result is in accordance with classical studies that report an increased insulin secretion from late pregnant rats (Green & Taylor 1972, Lipson & Sharp 1978). The upregulation of both the



**Figure 5** Effect of PRL and STAT3 knockdown on SERCA2 and SERCA3 expressions in RINm5F cells and insulin secretion in isolated pancreatic islets. CTL RINm5F cells and ASO transfected cells were transferred to a 1% BSA medium in the presence or absence of PRL. After 24 h, the cells were processed for western blot detection of SERCA2 (A;  $n=5$ ). Cells treated with PRL were also processed for western blot detection of SERCA3 (B;  $n=3$ ). Groups of 300 pancreatic islets from virgin female rats were cultured overnight and transiently transfected with antisense oligonucleotide against STAT3 (ASO). CTL islets were treated only with Lipofectamine 2000. After 36 h of culture, islets were used for insulin secretion assay (C;  $n=12$ ). The results represent the relative expressions of SERCA2 and SERCA3 as determined by optical densitometry, and the 1-h cumulative insulin secretion by RIE. Data are presented as mean  $\pm$  S.E.M. \* $P<0.05$  versus CTL; # $P<0.05$  versus PRL; @ $P<0.05$  versus glucose 2.8 mM within the same condition; & $P<0.05$  versus CTL in the presence of glucose 16.7 mM.

first and the second phases is no longer observed in early lactating rats. Indeed, the first phase was almost abolished after parturition. Whilst it has been previously reported that the insulin secretory response to glucose returns to control values during early lactation (Hubinont *et al.* 1986, Kawai & Kishi 1999), the dynamic insulin secretion pattern has not been

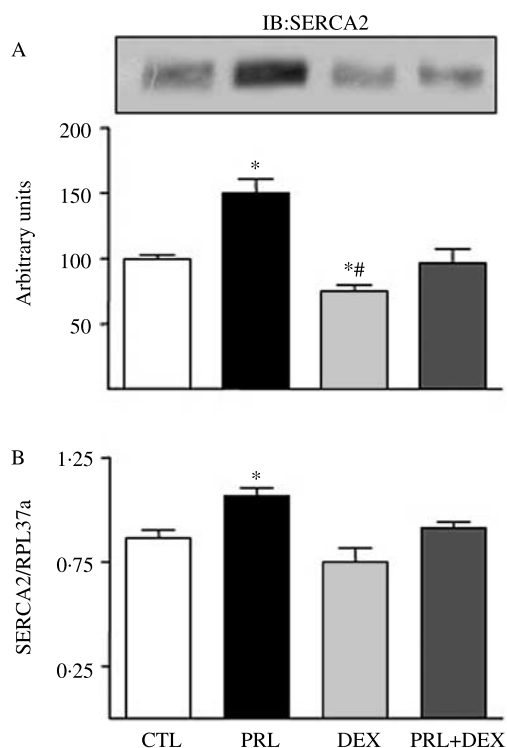
demonstrated. The return of the secretory response to a non-pregnant state is believed to occur due to a decrease in the rapid exchangeable islet calcium pool (Hubinont *et al.* 1986, Hubinont & Malaisse 1987). The increase in SERCA2, but not SERCA3, in islets from pregnant rats and its immediate reduction on day 3 of lactation might be a



**Figure 6** Effect of DEX and PRL on STAT3 expression, and serine and tyrosine phosphorylation in RINm5F cells. RINm5F cells were treated with PRL and DEX alone or combined (PRL + DEX) for 24 h. After the treatment, cells were processed for protein extraction and western blot detection of STAT3 (A), pSTAT3-Tyr (B), and pSTAT3-Ser (C). The bars represent the means  $\pm$  s.e.m. of the values determined by optical densitometry. <sup>\*</sup> $P < 0.05$  versus CTL; <sup>##</sup> $P < 0.05$  versus PRL;  $n = 7$ .

mechanism by which the reduction in intracellular calcium occurs in islets from lactating rats. This hypothesis is reinforced here by the significant decrease in the first phase of insulin secretion in pancreatic islets of P19 rats after the pharmacological inhibition of SERCA. Therefore, our results corroborate the former postulation of Hubinont & Malaisse (1987) that a depletion of endogenous calcium stores accounts, at least in part, for the decreased insulin secretory responsiveness to glucose in lactation. Additionally, our results suggest that calcium stores in pancreatic islets from pregnant rats, which might result from increased SERCA2 content, also play a role in the upregulation of the first phase of insulin secretion during late pregnancy.

Gorogawa *et al.* (2004) have shown that the disruption of the STAT3 gene in  $\beta$ -cells (STAT3-insKO mice) resulted in an impairment of the first phase of glucose-stimulated insulin secretion. It is well known that  $\text{Ca}^{2+}$  handling in pancreatic  $\beta$ -cells plays an important role in the regulation of insulin secretion. Diminished SERCA expression and activity has been described as a common dysfunction related to the poor insulin secretion observed in animal models of diabetes (Roe



**Figure 7** Effect of DEX and PRL on SERCA2 expression in RINm5F cells and pancreatic islets. RINm5F cells and islets were treated with PRL and DEX alone or combined (PRL + DEX) for 24 h. After the treatment, RINm5F cells were processed for protein extraction and western blot detection of SERCA2 (A), and pancreatic islets were processed for total RNA extraction and RT-PCR analysis of SERCA2 mRNA using RPL37a as housekeeping gene (B). The bars represent the means  $\pm$  s.e.m. of the values determined by optical densitometry. <sup>\*</sup> $P < 0.05$  versus CTL; <sup>##</sup> $P < 0.05$  versus PRL;  $n = 4$ .



*et al.* 1994, Varadi *et al.* 1996, Levy *et al.* 1998, Marie *et al.* 2001, Kulkarni *et al.* 2004). Although both SERCA2 and SERCA3 isoforms are co-expressed in human and rat islets and in the RINm5F  $\beta$ -cell line (Varadi *et al.* 1996), several studies have suggested a central role for SERCA3 in  $\beta$ -cell function. The absence of SERCA3, however, is insufficient to alter glucose homeostasis or impair insulin secretion (Arredouani *et al.* 2002a). Moreover, SERCA2 instead of SERCA3 is involved in resting  $[Ca^{2+}]_i$  regulation in  $\beta$ -cells, which implicates SERCA2 as the primary SERCA in physiological conditions (Varadi & Rutter 2002). Corroborating our data, Crepin *et al.* (2007) have recently demonstrated that the expression of SERCA2 is upregulated by PRL in immortalized epithelial prostatic cells.

In order to investigate whether this marked and reciprocal temporal pattern of STAT3 serine phosphorylation and SERCA2 expression observed *in vivo* was directly correlated to PRL effects on  $\beta$ -cell, we examined SERCA2 expression in the  $\beta$ -cell lineage, RINm5F, treated with PRL after the knockdown of STAT3 gene expression. Similarly to the study of Crepin *et al.* (2007), PRL also stimulated SERCA2 expression in RINm5F cells. STAT3 knockdown diminished SERCA2 expression in RINm5F cell and abolished PRL-induced SERCA2 expression, indicating that SERCA2 is regulated by STAT3. Additionally, PRL had no effect on SERCA3 expression. Our results also show that the knockdown of STAT3 expression induces, rather than inhibits, a significant increase in cumulative insulin secretion from pancreatic islets. This effect is in accordance with the dynamic insulin secretion of P19 islets treated with thapsigargin. The pharmacological blockage of SERCA induces a significant decrease in the first phase of insulin secretion but promoted a tendency to increase the second phase, which might result in an overall increase in the cumulative insulin secretion.

Although apparently contradictory, these results agree with several reports showing that SERCA pump inhibition results in an increase in the amplitude of the glucose-triggered  $Ca^{2+}$  transients, membrane depolarization, and insulin exocytosis (Arredouani *et al.* 2002a,b, Cruz-Cruz *et al.* 2005, Beauvois *et al.* 2006, Hughes *et al.* 2006). Moreover, in islets isolated from mice deficient in SERCA3 (the isoform predominantly expressed in mouse pancreatic  $\beta$ -cells), the glucose-stimulated insulin secretion is greater than those from the wild type (Arredouani *et al.* 2002a). Thus, we suggest that knockdown of STAT3, and by extension SERCA2, would decrease the first phase of insulin secretion (which corresponds to  $\sim 2\%$  of total amount of insulin secreted granules) and increase the second phase (corresponding to 98% of the total granules secreted). The overall response of this effect can be detected as an increase in cumulative insulin secretion during a 1-h assay.

In this way, we suggest that the STAT3-mediated SERCA2 expression, showed in the present study, is a mechanism by which PRL may contribute to the increased glucose-induced first phase of insulin secretion during pregnancy. Additionally, these data corroborate previous findings that have correlated

the loss of the secretory response in db/db mice with decreased SERCA activity (Roe *et al.* 1994). Importantly, the recovery of the secretory response in Zucker diabetic fatty rats (ZDF) by overexpression of functional leptin receptors occurs simultaneously to a striking increase in the STAT3 phosphorylation status (Wang *et al.* 1998).

The understanding of the mechanisms that regulate the early phase of glucose-induced insulin secretion is particularly relevant because subjects with type 2 diabetes show a defective dynamic of insulin release due to a preferential reduction on first-phase insulin secretion (reviewed by Ahrén 2005). The impairment of the first phase of insulin release frequently found in diabetic patients extends the duration of postprandial hyperglycemia and, as a consequence, stimulates a compensatory late-phase hyperinsulinemia (Caumo & Luzi 2004). Anatomically, the liver is the main target for first-phase insulin secretion. As the secreted insulin reaches the circulation through the portal system, a greater hepatic than peripheral insulinization is rapidly achieved (the so-called porto-systemic gradient). Thus, according to this hypothesis, the first phase of insulin secretion is an immediate event that prompts a significant suppression of hepatic glucose production (Luzi & DeFronzo 1989).

Whilst PRL promotes a gain-of-function in islets during pregnancy, at the end of the gestational period islets undergo a return to the normal non-pregnant function. The increased maternal GCs, a characteristic of the late stage of gestation, have been suggested to contribute to reduced glucose-stimulated insulin secretion, probably by counteracting the effect of PRL (Weinhaus *et al.* 2000, Shao *et al.* 2004). The striking correlation between STAT3 serine phosphorylation and SERCA 2 expression during the peripartum period and the fact that these alterations are likely to be the result of PRL action in pancreatic  $\beta$ -cells prompted us to investigate whether GCs could counteract this effect. The present results show that DEX abolished PRL-induced upregulation of STAT3 serine phosphorylation without affecting STAT3 expression. This effect was accompanied by the abolishment of the upregulation of SERCA2 expression by PRL, in both RINm5F cells and cultured pancreatic islets. Therefore, the negative modulation of the PRL-induced augmentation in STAT3 serine phosphorylation and SERCA2 expression might participate in the ability of GCs to counteract the biological effects of PRL in pancreatic islets. In agreement with this proposition, it was recently described that the pre-treatment with DEX reduces the first and the second phases of insulin secretion by rat islets perfused with glucose 15 mM (Zawalich *et al.* 2006). Therefore, we speculate that the downregulation of STAT3-mediated SERCA2 expression by GC might account for its ability to suppress the first phase of insulin release. The effect of GC in reducing the second phase of insulin secretion, as observed in islets from L3 rats, is probably due to a distinct mechanism.

In summary, the present study shows that STAT3 expression and serine phosphorylation are increased in pancreatic islets from pregnant rats. These events are probably

due to the action of PRL and may therefore upregulate SERCA2 expression, contributing to the increased pancreatic  $\beta$ -cell response to glucose, particularly modulating the first phase of insulin secretion. Moreover, PRL-induced STAT3 serine phosphorylation and SERCA2 expression are counteracted by GCs, suggesting an intracellular mechanism by which insulin secretion returns to normal after delivery.

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